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Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy

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Summary. Outbreaks of avian influenza due to an H7N1 virus of low pathogenicity occurred in domestic poultry in northern Italy from March 1999 until December 1999 when a highly pathogenic avian influenza (HPAI) virus emerged. Nucleotide sequences were determined for the HA1 and the stalk region of the neuraminidase (NA) for viruses from the outbreaks. The HPAI viruses have an unusual multibasic haemagglutinin (HA) cleavage site motif, PEIPKGSRVRRGLF. Phylogenetic analysis showed that the HPAI viruses arose from low pathogenicity viruses and that they are most closely related to a wild bird isolate, A/teal/Taiwan/98. Additional glycosylation sites were present at amino acid position 149 of the HA for two separate lineages, and at position 123 for all HPAI and some low pathogenicity viruses. Other viruses had no additional glycosylation sites. All viruses examined from the Italian outbreaks had a 22 amino acid deletion in the NA stalk that is not present in the N1 genes of the wild bird viruses examined. We conclude that the Italian HPAI viruses arose from low pathogenicity strains, and that a deletion in the NA stalk followed by the acquisition of additional glycosylation near the receptor binding site of HA1 may be an adaptation of H7 viruses to a new host species i.e. domestic poultry.

Introduction

Avian influenza A viruses infecting poultry can be at one or other of the two extremes of pathogenicity, causing inapparent to mild disease, or a devastating highly infectious disease with rapid mortality that often approaches 100%. Viruses that cause the latter disease, termed highly pathogenic avian influenza (HPAI) are

always of the H5 or H7 subtypes, although not all viruses of these subtypes cause HPAI. The natural reservoir for all avian influenza A viruses is wild birds, in particular waterfowl (especially Anseriformes and Chariidriformes species), but very few HPAI viruses have been isolated from this source.

Multiple basic amino acids at the cleavage site of the haemagglutinin (HA) glycoprotein are an absolute requirement for HPAI viruses [3, 15, 16, 21, 30]. However, the highly pathogenic phenotype has been shown to be polygenic [24, 25], and possibly modulated by an interrelationship between the number of glycosylation sites on the HA1 and neuraminidase (NA) stalk length [18]. The mechanisms by which highly pathogenic forms of avian influenza arise is not well understood.

Phylogenetic studies suggest that HPAI viruses do not form separate lineages from viruses of low virulence, and this supports the current theory that they arise from viruses of low pathogenicity [2, 23] possibly after introduction into domestic poultry [18]. There have been several documented examples of avian influenza viruses of low pathogenicity infecting poultry flocks before the emergence of HPAI strains [11, 14]. But only during the extended outbreaks in Mexico between 1994 and 1995 due to a virus of H5N2 subtype, were sufficient isolations of low pathogenicity viruses made prior to the emergence of the highly pathogenic strain, to allow the stepwise acquisition of virulence by an avian influenza virus in nature to be followed. A model for the generation of an insertion at the cleavage site was proposed in which purine nucleotides (A and G) are added due to slippage of the viral RNA polymerase [10]. No similar studies have been conducted for viruses of the H7 subtype.

In March 1999 an H7N1 virus of low pathogenicity for chickens was isolated from turkeys in the northern region of Italy [12]. Following this initial isolation, 199 outbreaks were reported and in December 1999 the first HPAI H7N1 virus was isolated. Between December 1999 and April 2000, a total of 413 HPAI outbreaks occurred in poultry in Italy.

In the present study we have analysed the molecular changes occurring in the HA1 and the NA of H7N1 avian influenza viruses isolated prior to and during a major outbreak of HPAI in poultry.

Materials and methods

The complete coding sequences for the HA1 of fifty-six H7N1 subtype influenza viruses isolated from poultry during avian influenza outbreaks in Italy between March 1999 and February 2000 were determined. The HA cleavage site motif was determined for a further 433 viruses isolated between March 1999 and September 2000. RNA extraction, reverse transcription (RT), polymerase chain reaction (PCR) and sequencing were done as described [1]. Partial sequences for the NA gene were determined for eight Italian isolates and four other N1 viruses held in the repository at VLA-Weybridge (Table 1) (primers available on request). All manipulations with infectious viruses were performed in a category III high containment laboratory. Additional published HA and NA sequences were obtained from GenBank. All virus strains with abbreviations, accession numbers and origin where appropriate, are listed in Tables 1 and 2. The Lasergene software package (Dnastar, Madison, USA) was used for the assembly, analysis and translation of nucleotide sequence

Table 1. The N1 neuraminidase stalk lengths, HA glycosylation status, species of origin and pathogenicity of selected avian influenza viruses

Virus	HA Subtype	Patho-genicity	Position of NA deletion	GenBank accession number	Additional HA glycosylation
A/FPV/Rostock/34	H7	HPAI	54–75	X52226	123
A/parrot/Northern Ireland/VF-73-67/73	H7	LP[a]	None	K02252	–
A/turkey/England/50-92/91	H5	HPAI	45–67		–
A/fairy bluebird/Singapore/F92/94	H7	LP	None		–
A/conure/England/766/94	H7	LP	None		–
A/goose/Guangdong/1/96	H5	HPAI	None	AF144304	123
A/Hong Kong/156/97	H5	HPAI	54–72	AF028708	–
A/duck/Hong Kong/p45/97	H5	HPAI	54–72	AF098552	?
A/duck/Hong Kong/y283/97	H5	HPAI	54–72	AF098553	?
A/goose/Hong Kong/w355/97	H5	HPAI	54–72	AF098554	?
A/chicken/Hong Kong/220/97	H5	HPAI	54–72	AF046081	?
A/chicken/Hong Kong/258/97	H5	HPAI	54–72	AF057292	158
A/chicken/Hong Kong/728/97	H5	HPAI	54–72	AF098548	–
A/chicken/Hong Kong/786/97	H5	HPAI	54–72	AF098549	158
A/chicken/Hong Kong/915/97	H5	HPAI	54–72	AF098550	–
A/waterfowl/Hong Kong/m603/97	H5	?	None	AF098551	?
A/teal/Taiwan/WB2-32-2TPFE2/98	H7	LP	None		–
A/chicken/Taiwan/7-5/99	H6	?	42–53	AF208598	?
A/turkey/Italy/977/99	H7	LP	54–75		–
A/turkey/Italy/2379/99	H7	LP	54–75		123
A/turkey/Italy/2505/99	H7	LP	54–75		149
A/turkey/Italy/3185/99	H7	LP	54–75		149
A/turkey/Italy/5563/99	H7	LP	54–75		–
A/turkey/Italy/4617/99	H7	HPAI	54–75		123
A/chicken/Italy/4756/99	H7	HPAI	54–75		123
A/turkey/Italy/4794/99	H7	HPAI	54–75		123

[a]Low pathogenicity

data. Phylogenetic analysis was performed for the first 1000 nucleotides of the HA1 coding region, by the maximum likelihood [7] or Fitch methods, using the PHYLIP phylogenetic inference package (version 3.57c) [8]. A transition/transversion ratio of 2.78 was calculated for the data set using PUZZLE [26] and used in the maximum likelihood analysis. The phylogenetic analysis is presented as an unrooted maximum likelihood phylogram, where the horizontal branch lengths are proportional to the nucleotide differences between the sequences (Fig. 1).

Evolutionary rates were calculated from an estimate of the number of substitutions (using the distance matrix method Fitch) occurring in isolates as compared with the first isolate (977), plotted against the date of sampling. The slope of the regression line equates to the rate of accumulation of mutations. All 56 viruses sequenced were used for these calculations.

The pathogenicity of viruses was inferred from HA cleavage site motifs or determined by intravenous pathogenicity tests for some viruses [4].

Table 2. Sampling data, species, geographical location and amino acid changes in the HA1 as compared with the consensus for the Italian viruses

Virus	Host species	Sampling date	Municipality	−10	−4	−1	11	47	48	56	58	60	68	82	94	95	101	104	109	112	125	126	128	130	151	155	177	188	196	201	208	209	210	249	298	303
Amino acid position				−10	−4	−1	11	47	48	56	58	60	68	82	94	95	101	104	109	112	125	126	128	130	151	155	177	188	196	201	208	209	210	249	298	303
Consensus				F	I	N	S	R	T	L	G	I	Q	E	S	E	I	E	D	T	A	T	A	R	A	M	G	S	G	Q	P	G	A	S	R	E
Amino acid change				L	T	S	P	K	A	I	T	T	R	G	R	G	V	G	E	A	T/V[a]	S	T/S*	K/E[a]	T	L	V	G	E	L	L	E	E/S*	I	K	Q
977	turkey	26/03/99	Erbè (VR)																										+							
1067	turkey	02/04/99	Nogarole Rocca (VR)																								B					B				
1081a	broiler breeder	02/04/99	Zimella (VR)				A														A		A													
1082	broiler breeder	02/04/99	Zimella (VR)																								B									
1083	turkey	02/04/99	Isola Rizza (VR)																			+														
1085	turkey	02/04/99	Isola Rizza (VR)																																	
1086	turkey	02/04/99	Nogara (VR)																																	
1174	broiler turkey	07/04/99	Porto Tolle (RO)																									+								
1265	turkey	07/04/99	Isola della scala (VR)																																	
1279	broiler chicken	09/04/99	Nogara (VR)																																	
4857a	broiler chicken	14/04/99	Trevenzuolo (VR)																		A													A		
1555	turkey	20/04/99	S. Martino Buonalbergo (VR)							B																	B									
4073d	turkey	28/04/99	Paderno (BS)																																	+
4499	turkey	10/05/99	Cazzago S. Martino (BS)																			+*														
4603	turkey	10/05/99	Ghedi (BS)					+																									+*			
2379a	turkey	04/06/99	Sommacampagna (VR)											A				A			A															
2505b	broiler turkey	06/07/99	Palù (VR)																						B		B									
2715b	turkey	02/08/99	Isola della scala (VR)																						B	B	B									
2716	turkey	02/08/99	Isorella (BS)																																	
5563d	turkey	03/08/99	Emilia Romagne																																	+
3185b	broiler turkey	02/09/99	Erbè (VR)									B													B	B	B	B			B					
3283c	broiler turkey	10/09/99	Verona (VR)						C							C									C											
3466a	turkey	20/09/99	Verona (VR)																		A									A						
3468	chicken breeders	20/09/99	Roverchiara (VR)														+																			
3489b	turkey	20/09/99	Sorgà (VR)	B								B													B	B	B	B			B					
3560c	turkey	27/09/99	Roverchiara (VR)										C			C					C*		C*	C	C											
3675b	turkey	04/10/99	Trevenzuolo (VR)									B													B	B	B	B			B				B	
3819	poultry	14/10/99	Roverè Veronese (VR)																																	
4042b	turkey	23/10/99	Veronella (VR)		B							B													B	B	B	B			B					
4294c	turkey	22/11/99	Nogarole Rocca (VR)										C		C	C					C*			C*	C											
4580a*	turkey	10/12/99	Salizzole (VR)																	A	A		A										A			
4620	poultry	13/12/99	Sanguinetto (VR)																																	
4644c	turkey	13/12/99	Villafranca di Verona (VR)										C		C	C					C*			C	C											
4640a*	turkey	15/12/99	Sanguinetto (VR)																	A	A		A										A			
4725	turkey	20/12/99	S. Martino Buonalbergo (VR)								+																									
4911a*	broiler	27/12/99	Lendinara (RO)			A														A	A		A										A			
5079a*	layers	30/12/99	Roverbella (MN)																A	A	A		A*										A			
5032a*	peacocks	31/12/99	Pernumia (PD)																	A	A		A										A			
5074a*	broiler	31/12/99	Idro (BS)																	A	A		A										A			
984a*	ostrich	04/02/00	Villafranca di Verona (VR)																	A	A		A										A			

(Amino acid changes)

+ No additional glycosylation. *A* Viruses from lineage with glycosylation site 123a. *B* Viruses from lineage with glycosylation site 149b. *C* Viruses from lineage with glycosylation site 149c. [a]Alternative substitution. *VR* Verona city, Veneto region. *RO* Rovigo Province, Veneto region. *BS* Brescia City, Lombardia Region. *MN* Mantova, Lombardia region. *PD* Padova, Veneto region. Vertical lines mark the glycosylation sites 123 and 149. * HPAI

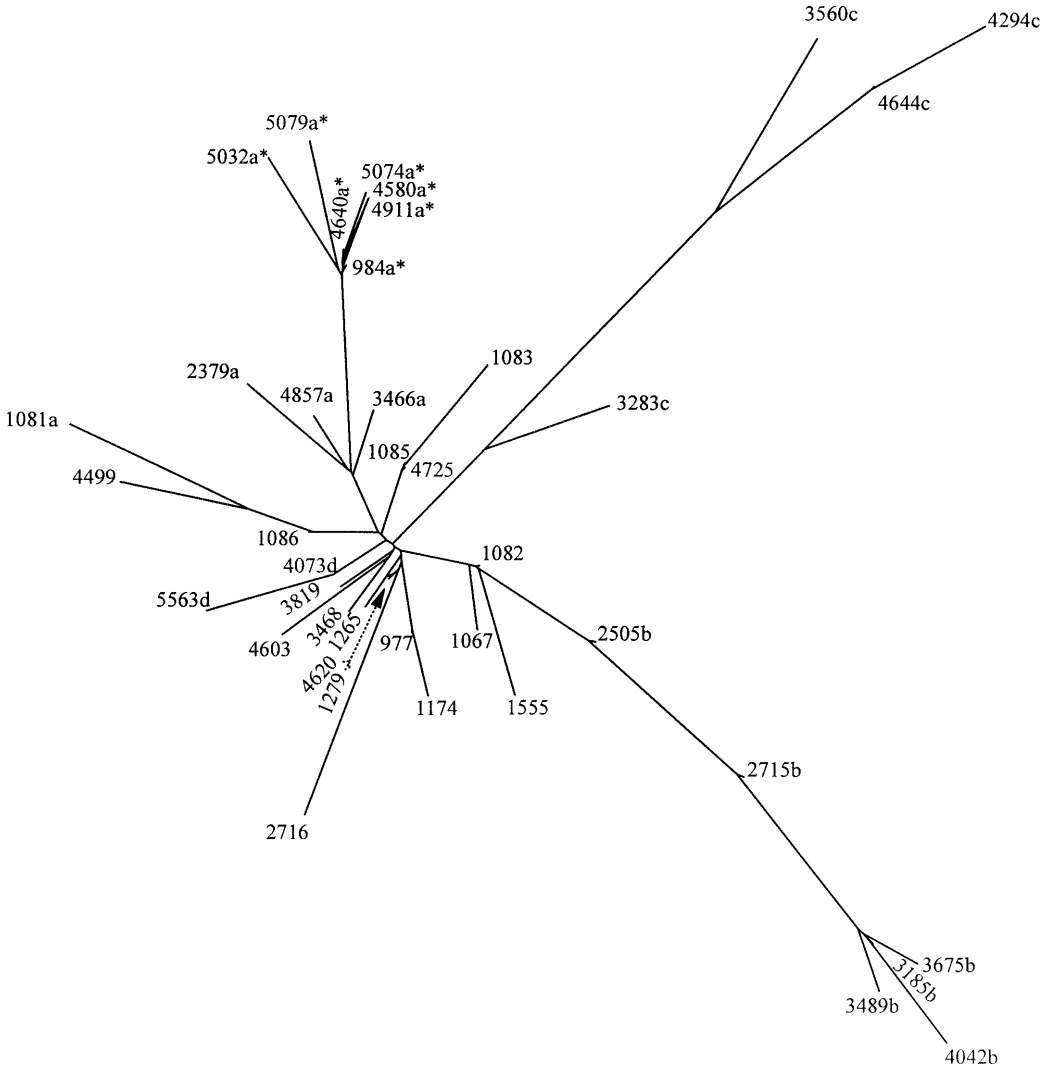

Fig. 1. Phylogenetic tree for 1000 nucleotides of the HA1 of the 1999–2000 Italian influenza viruses (maximum likelihood). The results are presented as an unrooted maximum likelihood phylogram, where the branch lengths are proportional to the nucleotide difference between the sequences. *a* viruses with glycosylation sequon 123, *b* and *c*, viruses with glycosylation sequon 149, *d* viruses with PEVPK cleavage site motif, * HPAI

Results

At the HA cleavage site, 9 nucleotide sequences coding for 5 different amino acid motifs were observed. The deduced amino acid cleavage site motif obtained for the 1999 Italian viruses of low pathogenicity was the expected European motif PEIPKGRGLF, except for 10 viruses that had the unusual motif of PEVPKGRGLF. Most of the HPAI (360/364) isolates had an insertion of the same four amino acids (underlined) giving the motif PEIPKGSRVRRGLF, whilst the alternative motifs PEIPKGSRMRRGLF and PEIPKRSRVRRGLF were observed in three and one virus respectively.

Forty different HA nucleotide sequences were obtained from the fifty-six viruses for which the entire HA1 was examined (GenBank accession numbers AF364133–AF364172). A total of sixty-three different nucleotide substitutions was observed when compared with the consensus sequence. Of these, thirty-seven were non-synonymous mutations at thirty-three sites (Table 2). The first 1000 nucleotides of the HA1 coding region of the forty unique sequences were used for the phylogenetic analysis; this eliminates any bias that would result from the inclusion of the variable cleavage sites (Fig. 1).

The viruses shared high HA gene nucleotide homologies, with identities (calculated from pair distances), between 98% and 100%. However, the phylogenetic analysis revealed three lineages, which appear to be emerging from a common pool of viruses that possibly originate from a single introduction to poultry from the wild bird reservoir. The distinguishing feature for viruses in all three lineages is the acquisition of an additional glycosylation sequon at the globular head of the HA1. Viruses marked with letter 'a' in Fig. 1 all share a glycosylation sequon at position 123 (H7 numbering). At the 'root' of this lineage the viruses are of low pathogenicity for chickens, by the end of the lineage all the viruses are HPAI. The rate of accumulation of mutations for this branch is 3.6×10^{-4} substitutions per nucleotide per year. Viruses in the other two lineages, although divergent, share the acquisition of a glycosylation sequon at position 149 (viruses marked 'b' and 'c' in Fig. 1), none are HPAI. The rate of accumulation of mutations for these branches is 2.3×10^{-2} and 1.8×10^{-2} substitutions per nucleotide per year respectively. One virus (1081) has a glycosylation site at position 123, but is not in the same lineage as all the other viruses with this glycosylation site. These mutations seem to have occurred independently suggesting that glycosylation confers a selective advantage upon these viruses in the poultry host.

Clustered around the divergence point for the lineages are viruses without additional glycosylation sites, again none are HPAI. They do however have a range of different nucleotide or amino acid changes consistent with a pattern of non-cumulative change.

Nucleotide sequences for the region spanning the NA protein stalk were obtained for eight viruses from the Italian outbreak and four from the avian influenza virus repository at VLA-Weybridge. These sequences were compared with those of thirteen avian and one human N1 subtype viruses obtained from GenBank. The Italian viruses were selected to represent viruses without additional glycosylation sites in the HA1: 977-99 (the earliest isolate) and 5563-99, viruses with glycosylation site 149: 2505-99 and 3185-99, and viruses with glycosylation site 123 of both low pathogenicity: 2379-99, and high pathogenicity: 4717-99, 4794-00 and 4756-00. All eight Italian viruses shared an identical twenty-two amino acid deletion in the NA stalk, corresponding to amino acids 54-75 as compared with the six viruses with full length stalks (Table 1). Other viruses with NA stalk deletions are nine viruses from the H5N1 HPAI outbreak in Hong Kong in 1997 including the Human virus A/Hong Kong/156/97, the HPAI viruses A/ty/Eng/50-92/91 (H5N1), and A/FPV/Rostock/34 (H7N1) and the low pathogenicity virus A/chicken/Taiwan/7-5/99 (H6N1), missing 19, 23, 22, and 12 amino acids respectively. It is

noticeable that all these viruses are highly pathogenic with the exception of ckTW 7-5-99 which is of an HA subtype (H6) not associated with HPAI viruses. All the viruses with full-length neuraminidase stalks are of low pathogenicity, with the exception of A/goose/Guangdong/1/96. Of the H7 viruses (other than the Italian viruses) A/FPV/Rostock/34 has HA glycosylation sites at position 123 and 149 whilst the other four (A/fairy bluebird/Singapore/F92/94, A/teal/Taiwan/WB2-32-2TPFE2/98, A/conure/England/766/94, A/parrot/Northern Ireland/VF-73-67/73) do not have any additional glycosylation sites in HA1.

Discussion

Phylogenetic analysis has shown the low pathogenicity Italian H7N1 virus 1081/99 to be most closely related to a wild bird isolate A/teal/Taiwan/WB2-32-2TPFE2/98 within a group of Eurasian and South African viruses isolated since 1994 [2]. Viruses from the recent Italian outbreaks do not have high homology with the H7 subtype virus A/gull/Italy/692-2/93 (H7N2) that was isolated during surveillance studies of waterfowl and gulls in the Tuscany region of Italy [2]. Nevertheless, most of the recent Italian outbreaks have occurred in the regions of Veneto and Lombardia where 65% of Italy's intensively reared poultry population is concentrated. Although most of the outbreaks resulted from secondary spread, this area in northern Italy is on a main migratory flyway for waterfowl and these birds are probably the source for the initial introduction of low pathogenicity virus to poultry.

Three different amino acid motifs were observed at the HA cleavage site for the HPAI viruses. All three had four additional amino acids as compared with the motifs for viruses of low pathogenicity. The addition of serine and valine or methionine is unusual and could not occur by the proposed "viral RNA polymerase slippage" mechanism for the emergence of HPAI viruses [10]. Also the first additional arginine in the insert has a codon usage (CGT or CGC) that has never before been observed at the HA cleavage site for either H7 or H5 influenza viruses [10].

All the 1999/2000 Italian H7N1 influenza viruses have the three glycosylation sites conserved among H7 viruses at amino acid positions 12, 28 and 231 (H7 numbering). Some have an additional glycosylation site at either amino acid position 123 or 149, both these sites lie close to the receptor binding site in the globular head of the HA. These glycosylation patterns are reflected in the phylogenetic analysis, where three branches corresponding to the acquisition of glycosylation sites appear to diverge from a common ancestor (Fig. 1). Viruses with a glycosylation sequon at position 149 account for two branches, acquisition of this mutation could have occurred independently or at an early stage followed by divergence. The evidence supports the former because the 'roots' and early isolates of both phylogenetic branches do not share the 149 sequon. In addition, virus 1081 has the 123 glycosylation sequon but is not found within that lineage. When the date of sampling, glycosylation status/phylogeny and geographical area of isolation are considered together (Table 2 and Fig. 1), it is evident that the separate lineages

evolved contemporaneously and at diverse locations within the Veneto region (the majority of samples were within a 45 km radius). This might suggest that there is a selective pressure in the poultry host for acquisition of glycosylation sites in the proximity of the receptor binding site, and that these mutations occur relatively readily. Viruses with the glycosylation sequon at position 123 progress through time from low to high pathogenicity. The HPAI viruses were isolated from a variety of birds (including two backyard flocks, ostriches and pheasants) in distant areas, with the geographical spread probably aided by the panic that ensued among the poultry industry.

Viruses without additional glycosylation sites are all concentrated around the divergence point of the three main lineages. This is consistent with a pattern of non-cumulative mutations soon after the initial virus introduction into poultry followed by divergence into lineages when the mutations become fixed, as described by Fitch et al. [9] for the antigenic sites of influenza A viruses. It has previously been observed that contrary to the evolutionary stasis seen among influenza A viruses in feral birds [29], introduction of H5 subtype viruses to poultry can be followed by rapid genetic drift [11, 18] and the introduction of additional glycosylation sites is a feature of this drift. These findings are echoed with the H7 viruses in this study. Mutation and evolutionary rates for the lineages that have acquired additional glycosylation sites are comparable to those observed during the Mexican H5N2 outbreaks [11, 27]. Caution should be exercised when quoting and comparing mutation rates, because many variant genomes are produced during natural infections by RNA viruses. The dynamic mutant distributions that compose replicating RNA viruses are termed viral quasispecies, and sequence information derived from primary virus isolates represent the dominant or average sequence of that quasispecies population. Variations in the average nucleotide sequence reflect shifts in the equilibrium of quasispecies, caused by changing conditions, rather than changes in mutation rates [6]. Thus mutation and variation may be entirely different concepts [5]. The observed high mutation rates for influenza viruses in poultry hosts are most likely measuring variation due to changes in quasispecies equilibrium as an adaptation to a new host.

The glycosylation sites 123 and 149 are located adjacent to the receptor-binding site of HA and may regulate the affinity of the receptor-binding site for its receptor such that HA is able both to bind and to dissociate. For the HPAI virus A/FPV/Rostock/34 an increase in glycosylation decreases the affinity of the influenza virus HA for its receptor with 149 having the dominant regulatory effect. In addition, close co-operation is required between the HA and NA for virus entry and exit [19] terminal neuraminic acid residues must be removed from the oligosaccharides adjacent to the receptor-binding site before attachment of the HA to the receptor can occur [19, 20, 28]. Changes in glycosylation at the sites adjacent to the receptor-binding site often occur, and are dependent upon the host system in which the virus is propagated [13, 20, 22]. Thus by modulating receptor specificity and receptor avidity, glycosylation near the receptor binding site may be an important determinant for cell tropism and host range. Viruses from the Italian avian influenza outbreaks all have a twenty-two amino acid deletion in the

NA stalk that removes three potential glycosylation sites (Table 1). The effect this may have upon NA function is not clear, but viruses with different stalk lengths have been shown to have different growth characteristics in different host cells [17]. In addition, reduced NA activity due to a stalk deletion can be balanced by additional glycosylation of the HA near the receptor binding site [28]. All the viruses with NA stalk deletions probably had a domestic poultry origin whilst those with full-length stalks were isolated from wild or captive cage birds. The origin of the latter is not always known, however most have been wild or been in contact with wild birds. It is likely that a deletion in the NA stalk followed by the acquisition of additional glycosylation at the receptor binding site of the HA, in the Italian viruses, is an adaptation to a new host i.e. domestic poultry. Whether these changes also influenced the subsequent change to high pathogenicity is unknown. The HPAI virus A/goose/Guangdong/1/96 has a long NA stalk with a 123 glycosylation site HA phenotype, and the H6 virus chicken Taiwan 99 has a NA deletion suggesting that the two mutational events are probably unrelated. However, although not a prerequisite there does seem to be a preference for viruses with the 123 phenotype to become HPAI.

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